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- (71) Applicant (for all designated States except US): MARS UK LIMITED [GB/GB]; 3D Dundee Road, Slough, Berkshire SL1 4LG (GB).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): BAILLON, Marie-Louise [GB/GB]; Waltham Centre for Pet Nutrition, Waltham-on-the-Wolds, Leicestershire LE14 4RT (GB). GIFFARD, Catriona, Julie [GB/GB]; Waltham Centre for Pet Nutrition, Waltham-on-the-Wolds, Leicestershire LE14 4RT (GB).
- (74) Agents: CORNISH, Kristina, Victoria, Joy et al.; Kilburn & Strobe, 20 Red Lion Street, London WC1R 4PJ (GB).
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(54) Title: TREATMENT OF INFECTION IN ANIMALS

(57) Abstract: The present invention relates to the use of a non-digestible carbohydrate in the manufacture of a composition for treating or preventing pathogenic bacteria in the large intestine of a pet animal. It also provides a method for the prevention or treatment of a pathogenic bacteria in the large intestine of a pet animal, the method comprising administering to said pet animal a composition which comprises a non-digestible carbohydrate.

### TREATMENT OF INFECTION IN ANIMALS

5 The present invention relates to the use of a non-digestible carbohydrate in the manufacture of a composition for treating or preventing pathogenic bacteria in the large intestine of a pet animal. It also provides a method for the prevention or treatment of a pathogenic bacteria in the large intestine of a pet animal, the method comprising administering to said pet animal a composition which comprises a non-  
10 digestible carbohydrate.

Presence of pathogenic bacteria (including infection) of the large intestine in a pet animal is concerning. Particular pathogenic bacteria which infect the large intestine include *Campylobacter* and pathogenic *Escherichia coli*. The bacterial species  
15 responsible for the majority of human bacterial gastrointestinal infections is *Campylobacter jejuni*. This species is also the main cause of concern for cats and dogs. The species can act as a pathogen in young dogs and cats and is likely to be opportunistic in older animals. Clinical illness in dogs manifests itself as diarrhoea ranging from mild to mucus laden bloody diarrhoea, tenesmus, vomiting, anorexia and  
20 depression.

A major concern regarding *Campylobacter* infection in pet animals is the zoonotic risk which carriage and excretion of the organism represents. It has been estimated that 5% of all human *Campylobacter jejuni* induced diarrhoea results from exposure to  
25 infected cats or dogs. A number of more recent studies quote dog ownership as a significant risk factor for becoming ill with *Campylobacter*. A study carried out in Christchurch, New Zealand found that household contact with dogs carried a risk of 1.25 to 2 times normal for contracting *Campylobacter*.

*E. coli* is a Gram negative facultative anaerobic bacillus. In general it leads a symbiotic life with its host causing it no harm. However specific groups are known to cause gastrointestinal disease and are classified into categories as defined by their virulence mechanisms. Enteropathogenic and verocytotoxigenic strains of *E. coli* are particularly important in causing acute and chronic diarrhoea in dogs. The verocytotoxigenic *E. coli* are thought to be important in diarrhoeic as well as healthy cats and these animals are likely to act as a reservoir of infection for humans.

*Salmonella* organisms are Gram negative, facultative anaerobic bacteria that are able to survive intracellularly. *Salmonella* can cause clinical and subclinical infections in dogs and cats as well as humans. This makes them a key organism of interest.

Various studies have found *Salmonella* to be carried by between 1 and 30% of healthy domestic pet dogs and between 1 and 18% of healthy domestic pet cats. This data is dependent on the survey and whether *Salmonella* could be cultured from the faeces of animals both with and without diarrhoea.

Clinical infections of *Salmonella* in animals often display signs of mild to severe gastroenteritis. In dogs, symptoms most often reported are diarrhoea, vomiting, fever, malaise, anorexia, vaginal discharge and sometimes abortion. In cats, diarrhoea, vomiting, fever, malaise and anorexia are the predominant symptoms reported. Recovery from acute Salmonellosis typically occurs within 1 week but can take up to 3 to 4 weeks. Shedding of *Salmonella* in faeces can continue for 3 to 6 weeks and can be a source of infection for human family members.

Due to the long term shedding of *Salmonella*, animals are important vectors for non-foodborne infections in humans. Dogs have a greater zoonotic potential than cats although cats have been shown to shed organisms orally, conjunctivally and faecally.

Contact with faeces from infected pets is an important source of infection to young children.

5 The location of *Salmonella* infection is in the small intestine but due to the potential zoonotic risk from long term shedding of *Salmonella* in faeces from dogs, a model of the large intestine has been used for this investigation. If viable *Salmonella* numbers can be reduced while in the large intestine then the duration of shedding can be reduced. Reducing the time that *Salmonella* is shed in dog faeces also reduces the chance of human contact with the pathogen.

10 Accordingly, there is a need for a means of preventing or treating pathogenic bacteria in the large intestine of pet animals to eliminate the aforementioned risks. Current treatments for *Campylobacter* and pathogenic *E. coli* infections and the presence of *Salmonella* involve administering antibiotics to the pet animals. There are concerns  
15 that the continued use of antibiotics in treating *Campylobacter* infections may lead to the emergence of antibiotic resistant strains of this organism and may have an effect on the long-term health of pet animals (which would reduce or eliminate treatment options). A need therefore exists to find an alternative to antibiotic treatment for infected or infectable animals. The present invention provides such a means.

20 According to the present invention there is provided the use of a non-digestible carbohydrate in the manufacture of a composition for the prevention or treatment of pathogenic bacteria in the large intestine of a pet animal.

25 In general, non-digestible carbohydrates comprise those compounds which are not digested by mammals but may be metabolised by intestinal bacterial species belonging to the normal microflora for example, bifidobacteria and lactobacilli.

5 The term "non-digestible carbohydrate" includes oligosaccharides such as fructooligosaccharide (FOS), galactooligosaccharide (GOS), lactosucrose, maltooligosaccharide, xylooligosaccharide, inulin, fractionated inulin, and raffinose, as well as a dietary fibre component such as coconut endosperm fibre, beet pulp (such as sugar beet pulp), chicory (including chicory pulp), rice bran, carob bean or gum talha.

10 Oligosaccharides are naturally occurring compounds which can be found in a variety of fruits and vegetables such as bananas, tomatoes, artichokes, onions, garlic and cereals (eg wheat and barley). There are three varieties of FOS: 1-kestose, nystose, and B-fructofuranosylnystose. While FOS can be extracted from plants such as those mentioned above, they can also be formed artificially by adding one, two or three fructose units to a sucrose molecule by a B-(2-1)-glycosidic linkage of the fructose unit(s) to the fructose unit of sucrose. Similar artificial linking can be used to  
15 synthesize GOS and other oligosaccharides. FOS and GOS are synthetically made and sold. A single or multiple oligosaccharide may be used. One or more may be from a natural source or may be synthetic.

20 A single or multiple dietary fibre component may be used. The dietary fibre component may comprise or be derived from one or more of: coconut endosperm fibre, beet pulp, chicory, citrus pulp, rice bran, carob bean or gum talha. A single or multiple dietary fibre component may be used in combination with a single or multiple oligosaccharide.

25 Fresh coconut endosperm is an example of a dietary fibre component of the present invention. It has a typical nutrient distribution of water (35%), oil (44%), protein (6%), sugars (7%), fibre (3%) and ash (1%). However, the form of the coconut endosperm fibre for use according to all aspects of the present invention is not

limiting. The coconut endosperm fibre may be fresh or in any other form such as copra, defatted copra (also referred to, amongst others, as copra cake, copra presscake or copra meal) coconut flour, defatted coconut flour, full or defatted desiccated coconut, copra, or degraded coconut endosperm which has been heated or enzymatically treated.

Copra is a particularly suitable source of coconut endosperm fibre for use according to the present invention. Copra is dried coconut endosperm (usually sundried). Defatted copra is also particularly suitable. Defatted copra is the typical result of coconut endosperm which has been dried and had the coconut oil mechanically removed. Defatted copra cake is obtained by first obtaining copra, then crushing the copra through a press or expeller to remove most of the oil. The residue remaining is termed copra cake, copra presscake, or copra meal.

One of the products of complex carbohydrate fermentation in the large intestine by normal microflora are short chain fatty acids (SCFAs). The production of SCFAs by the gut microflora results in a decrease in the pH of the lumen of the large intestine/colon. However, not all intestinal bacteria can metabolise non-digestible carbohydrates. Studies show that pathogenic bacteria are unable to process non-digestible carbohydrates. For this reason these non-digestible carbohydrates in the gut are involved in selectively stimulating the growth of the gut microflora creating a healthier gastrointestinal environment.

The non-digestible carbohydrate of the invention may be one which (additionally) selectively stimulates the growth and/or activity of one or a limited number of beneficial bacteria in the large intestine of a pet animal. The term "beneficial bacteria" refers to those species of bacteria which have a beneficial effect on the host organism.

The beneficial species of bacteria typically comprise one or more of bifidobacteria and lactobacilli.

5 A non-digestible carbohydrate of the present invention may be a "prebiotic". A prebiotic is defined in the art as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that have the potential to improve health.

10 Embodiments of the present invention have been shown to have potential in lowering the pH of the large intestine. Using an oligosaccharide, a lowering of the pH in the large intestine model from 7.25 to 5.5 was observed. In the large intestine, a lowering of the pH affects the survival of pathogenic bacteria. In the present invention, a lowering of pH from 7.25 to 5.5 as a result of the inclusion of FOS and/or GOS, is sufficient to eliminate viable *Campylobacter jejuni* bacteria. In contrast, the inclusion  
15 of coconut endosperm fibre in the large intestine results in the elimination of viable *Campylobacter jejuni* bacteria without a significant change in the pH of the large intestine (the pH was lowered from 7.0 to 6.5 in the large intestine model).

20 The present invention provides feeding pet animals with a composition comprising a non-digestible carbohydrate to reduce or eliminate viable pathogenic bacteria in the large intestine.

The pathogenic bacteria of the present invention typically include one or more of *Campylobacter*, pathogenic *Clostridium*, *Salmonella* and pathogenic *Escherichia coli*,  
25 such as verocytotoxigenic *E.coli*, for example *E. coli* O157. Of particular interest for the present invention are the species *Campylobacter jejuni* and *Salmonella enterica* (including *S. enterica* serotype Typhimurium). *Campylobacter jejuni* may cause

clinical illnesses including diarrhoea, tenesmus, vomiting, anorexia, depression, inflammatory intestinal disease and other intestinal disorders.

5 The pet animal of the present invention is preferably a mammal, most preferably a mammal having a single stomach as seen in a dog or a cat. The pet animal is preferably not a ruminant and not a production animal (suitable for meat production). The pet animal is preferably a cat or a dog. Cats and dogs according to the present invention are preferably *Felis domesticus* or *Canis domesticus*.

10 The composition for the prevention or treatment of a pathogenic bacteria in the large intestine of a pet animal is preferably a pet food product. The form or type of the pet food product is not limiting. It may be packaged. In this way, the consumer is able to identify, from the packaging, the ingredients in the food product and confirm that it is suitable for the particular pet animal in question. The packaging may be metal  
15 (usually in the form of a tin or flexifoil), plastic, paper or card. The pet food product may be a dry, semi-moist or a moist (wet) product. Wet food includes food which is sold in tins and has a moisture content of 70 to 90%. Dry food includes food having a similar composition, but with 5 to 15% moisture and presented as small biscuit-like kibbles. Semi-moist products have a moisture content between wet and dry products.  
20 The moisture content is in the range of from 15 to 70%. The amount of moisture in any product may influence the type of packaging which can be used or is required.

In combination with the non-digestible carbohydrate, the remaining components of the pet food product are not essential to the invention and typical standard products can be  
25 combined with the required non-digestible carbohydrate. Most preferably, the combined ingredients of the pet food product according to the invention provide all of the recommended vitamins and minerals for the particular pet in question, (a complete and balanced food), for example, as described in National Research Council, 1985,



Nutritional Requirements for Dogs, National Academy Press, Washington D.C. (ISBN: 0-309-03496-5); National Research Council, 1986, Nutritional Requirements of Cats, National Academy Press, Washington D.C. (ISBN: 0-309-03682-8) or Association of American Feed Control Officials, Official Publication 1996.

5

The pet food product according to the present invention encompasses any product which a pet consumes in its diet. Thus, the invention covers standard food products as well as pet food snacks (for example, snack bars, pet chew, crunchy treat, cereal bars, snacks, biscuits and sweet products). The food product is preferably a cooked product.

10

It may be in the form of a gelatinized starch matrix. It may be in the form of chunks in gravy, jelly, loaf or water. It may incorporate meat or animal derived material (such as beef, chicken, turkey, lamb, pork, fish, blood plasma, marrow bone etc or one or more thereof). The product alternatively may be meat free (preferably including a meat substitute such as soya, maize gluten or a soya product) in order to provide a protein source. The product may contain additional protein sources such as soya protein concentrate, milk proteins, gluten etc. The product may also contain a starch source such as one or more grains (e.g. wheat, corn, rice, oats, barley etc), or may be starch free. A typical dry dog or cat food contains about 20-30% crude protein and about 10-20% fat, the remainder being carbohydrate, including dietary fibre and ash. A typical wet, semi-wet or moist product contains (on a dry matter basis) about 40% fat (from 20 to 50%), 50% protein (from 40 to 60%) and the remainder being fibre and ash.

15

20

The pet food product is preferably a commercial pet food product. Such a product is preferably sold as a product for feeding to a pet animal, in particular a pet cat or a pet dog.

25

The pet food product may comprise from 0.1 to 5 weight% of an oligosaccharide, most preferably from 0.1 to 2 weight% (on a dry matter basis).

5 The level of non-digestible fibre incorporated into a pet food product such as a dietary fibre component is not limiting. Preferably, the fibre component is present in the pet food product at a level of from approximately 0.15 to 8% on a dry matter basis, preferably 0.15 to 5% on a dry matter basis as measured by the Englyst method (as defined in Englyst H.N., and Cumming J.H. (1984), simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst*. 109, 937-942, and incorporated herein by reference). The levels, as calculated by this method, may go from 0.15% up to 5%, 6%, 7% or 8%. The lower limit may be from 1.5%, 2% or 3%. A description of the Englyst method is described in Appendix 1. In principle, starch is removed enzymatically after solubilisation and NSP is measured as the sum of the constituent sugars released by acid hydrolysis. The starch component of the fibre source is gelatinised by boiling in hot water and is then removed with  $\alpha$ -amylase and pullulanase. Starch and modified, or resistant starch are dispersed in DMSO. Three samples are then subjected to complementary procedures measuring (i) total NSP (ii) water-soluble NSP and (iii) cellulose. Components are hydrolysed in each case with sulphuric acid. The constituent sugars are converted to alditols and are measured as their alditol acetates using gas-liquid chromatography (GLC). Values for total dietary fibre as well as insoluble and soluble fractions can be obtained. Cellulose can be measured separately and the non-cellulose polysaccharides are characterised by measurement of the individual monosaccharides.

25

The incorporation of the level of coconut endosperm fibre as an example of dietary fibre according to the invention (which may differ according to the form of the coconut endosperm, for example copra or desiccated coconut) can easily be

determined by identifying the amount of dietary fibre in the particular form of the coconut endosperm fibre. For example, according to the Englyst method (*Supra*) defatted copra contains approximately 33.5% total dietary fibre. Accordingly, the preferred amount of defatted copra in a pet food product in order to provide a preferred fibre level of from approximately 0.15 to 5% on a dry matter basis according to the first aspect of the invention is at a level from approximately 0.5 to 15% on a dry matter basis of the pet food product.

Without limiting the present invention, the addition of a non-digestible carbohydrate into a pet food product is believed to maintain good health of the large intestine or improve it, generally achieved by optimising the conditions for growth and multiplication of non-harmful bacteria in the large intestine and/or by lowering the pH of the lumen of the large intestine thereby reducing the amount of harmful bacteria in the large intestine.

A second aspect of the invention provides a method for the prevention or treatment of pathogenic bacteria in the large intestine of a pet animal, the method comprising administering to said pet animal a composition which comprises a non-digestible carbohydrate. The administration is preferably feeding, most preferably feeding by mouth.

Preferred features for the second aspect of the invention apply as for the first aspect *mutatis mutandis*, such as preferred non-digestible carbohydrates, preferred levels in a pet food product, preferred animals and relevant pathogenic bacteria.

In the second aspect of the invention, the method is preferably administered to a pet animal in need of the prevention or treatment of a pathogenic bacteria in its large intestine. This may be to, for example a young pet animal, such as a puppy, or an older

pet animal. Where the composition is a pet food product, the pet food product may be administered in a dietary regime in accordance with the usual dietary regime of the pet animal. The pet food product may comprise 100% of the diet of the pet animal or a lesser proportion, depending on the level of prevention or treatment required. The pet food product allows the non-digestible carbohydrate to be administered with ease thus avoiding a need to supplement the pet animal's food. In addition the pet food product can be administered by the animal's owner thus avoiding constant veterinary supervision. The pet food product may be available at any outlet selling pet food products or may be available from a veterinarian. The pet food product may be as described above according to the first aspect of the invention.

As used herein, the term "administration" also includes feeding or any other method of oral administration. Other means of administration may include tablets, capsules, injection, suppositories or any other suitable means.

A third aspect of the present invention provides a process for the manufacture of a pet food product as defined herein comprising mixing together ingredients with the non-digestible carbohydrate and optionally forming a pet food product. Heating/cooking may be applied to any one or more of the ingredients or non-digestible carbohydrate prior to, during or following the mixing.

Preferred features for the third aspect of the invention apply as for the first or second aspect *mutatis mutandis*.

The invention will now be described with reference to the following non-limiting figures, in which:

Figure 1 is a graph of *C. jejuni* cells at 0 and at 24 hours in incubation with or without coconut endosperm fibre.

5 Figure 2 is a graph in a graph of *S. typhimurium* cells at 0 and at 24 hours in incubation with or without GOS.

The invention will now be described with reference to the following, non-limiting examples:

10 **EXAMPLE 1**

The effect of fructooligosaccharide (FOS) on the survival of *Campylobacter jejuni* in a model of the canine intestine.

15 **METHOD**

1. *Campylobacter jejuni* cells were grown from stock cultures and cultured at 37°C under microaerobic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>). Liquid cultures were grown in 20ml volumes in 50ml conical flasks shaken on an orbital shaker.  
20 Overnight cultures grown in Mueller Hinton (MH) broth (Oxoid) were adjusted to A<sub>600</sub> 1.0 before inclusion in the assay.

2. Flasks were set up with 200ml MH broth, 1ml of the adjusted *Campylobacter jejuni* culture, and 2g fresh faeces. To test flasks, 1.4g FOS (CoSucra) was added and  
25 swirled to mix. Control flasks had no further additions.

3. Flasks were sampled at the start (0 hours) and end (24 hours) of the experiment to determine viable counts of *Campylobacter jejuni* cells by serially diluting samples

from the flasks and plating dilutions onto *Campylobacter* selective agar (LabM). Plates were incubated microaerobically for 48 hours and which, viable numbers were determined.

- 5      4.      At the end of the experiment the pH of the mixture in each flask was determined using Multistix (Bayer).
- 10      5.      The experiment was conducted six times using a faecal sample from a different dog each time. All dogs were fed a complete and balanced dry pet food formula for the duration of the study.

## RESULTS

- 15      After a 24 hour microaerobic incubation, no viable *Campylobacter jejuni* cells could be recovered from flasks that had FOS added. In contrast, *Campylobacter jejuni* cells were recovered from the flasks that contained no FOS at around  $10^7$  cells per ml. The results from the 6 individual experiments are shown in Table 1.

**Table 1** Numbers of viable *Campylobacter jejuni* cells ( $\log_{10}$ ) recovered from the model of the canine large intestine with and without the addition of FOS.

←  $\log_{10}$  colony forming units of *Campylobacter jejuni* →

Faecal Sample	0 hours + 0.7% FOS	0 hours NO FOS	24 hours + 0.7% FOS	24 hours NO FOS
GR 4	7.38	7.41	0	7.55
L1525	7.57	7.7	0	8.26
L1544	7.48	7.42	0	7.6
L1508	7.19	7.13	0	7.03
L1512	7.3	7.33	0	8.12
L336	7.52	7.36	0	7.89
Mean	7.41	7.39	0	7.74
<u>STD</u>	0.14	0.18	0	0.45

At the end of the incubation period the pH of the solutions in each flask was measured and was found to be approximately 7.25 when FOS was omitted from the system. When FOS was included in the model, the pH was found to be approximately 5.5.

### CONCLUSIONS

Inclusion of FOS in a model of the canine large intestine resulted in the elimination of viable *Campylobacter jejuni* cells. With no FOS added to the system, *Campylobacter jejuni* cells were able to survive for the duration of the experiment with no fall in

**Table 2** Numbers of viable *Campylobacter jejuni* cells ( $\log_{10}$ ) recovered from the model of the canine large intestine with and without the addition of GOS.

$\leftarrow \log_{10}$  colony forming units of *Campylobacter jejuni*  $\rightarrow$

Faecal	0 hours	0 hours	24 hours	24 hours
Sample	+ 0.7% GOS	NO GOS	+ 0.7% GOS	NO GOS
GR1	7.28	7.23	0	8.71
L1550	7.42	7.2	0	8.68
L136	7.15	7.09	0	8.88
L1526	7.06	7.13	0	7.22
L1515	7.58	7.59	0	8
GR3	7.11	7.18	0	7.3
Mean	7.27	7.24	0	8.13
STD	0.2	0.18	0	0.74

5

At the end of the incubation period the pH of the solutions in each flask was measured and was found to be approximately 7 when GOS was omitted from the system (SD of 0.1). When GOS was included in the model, the pH was found to be approximately 5 (SD of 0.26).

10

### CONCLUSIONS

15

Inclusion of GOS in a model of the canine large intestine resulted in the elimination of viable *Campylobacter jejuni* cells. With no GOS added to the system, *Campylobacter*



5     *jejuni* cells were able to survive for the duration of the experiment. It is likely that the difference in pH observed between the two conditions was responsible for the difference observed in survival. It is likely that the non-pathogenic, saccharolytic bacteria present in the faeces metabolise the GOS and produce SCFA that decrease the pH in the model of the large intestine used in this study and this decrease in pH cannot be tolerated by *Campylobacter*.

### EXAMPLE 3

10     The effect of coconut endosperm fibre on the survival of *Campylobacter jejuni* in a model of the canine intestine

### METHOD

15     Investigation into the effect of coconut endosperm fibre on the survival of *Campylobacter* in the canine intestine.

### Summary

- 20
- *Campylobacter* is one of the most predominant gastrointestinal pathogens causing both clinical and non-clinical infections in dogs.
  - An *in vitro* model of the canine large intestine has been developed to test the effect of novel fibres on the survival of canine bacterial pathogens.
  - Inclusion of coconut endosperm fibre in this model resulted in the elimination of viable *Campylobacter jejuni* cells from the system.
- 25

### Methods

1. *Campylobacter jejuni* cells were grown from stock cultures and cultured at 37°C under microaerobic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>). Liquid cultures were grown in 20ml volumes in 50ml conical flasks shaken on an orbital shaker. Overnight cultures grown in Mueller Hinton (MH) broth (Oxoid) were adjusted to A<sub>600</sub> 1.0 before inclusion in the assay.
2. Flasks were set up with 200ml MH broth, 1ml of the adjusted *Campylobacter jejuni* culture, and 2g fresh faeces. To test flasks, 0.7% (w/v) copra cake was added and swirled to mix. Control flasks had no further additions.
3. Flasks were sampled at the start (0 hours) and end (24 hours) of the experiment to determine viable counts of *Campylobacter jejuni* cells by serially diluting samples from the flasks and plating dilutions on to *Campylobacter* selective agar (LabM). Plates were incubated microaerobically for 48 hours, after which viable numbers were determined.
4. At the end of the experiment the pH of the mixture in each flask was determined using Multistix (Bayer).
5. The experiment was conducted six times using a faecal sample from a different dog each time. All dogs were fed a commercially available premium (complete and balanced) dry food for the duration of the study.

### RESULTS

After a 24 hour microaerobic incubation, no viable *Campylobacter jejuni* cells could be recovered from flasks that had coconut endosperm fibre added. In contrast, *Campylobacter jejuni* cells were recovered from the flasks that contained no coconut

endosperm fibre at around  $10^8$  cells per ml. The results from the 6 individual experiments are shown in the table below and in the graph in Figure 1.

5 **Table 3** Numbers of viable *Campylobacter jejuni* cells ( $\log_{10}$ ) recovered from the model of the canine large intestine with and without the addition of coconut endosperm fibre.

<----- $\log_{10}$  colony forming units of *Campylobacter jejuni*----->

Faecal Sample (Dog No)	0 hours < +0.7%	0 hours Coconut None	24 hours Endosperm +0.7%	24 hours Fibre > None
1	7.76	7.75	0	7.25
2	7.33	7.64	0	8.56
3	7.77	7.75	0	7.17
4	7.72	7.74	0	7.47
5	7.67	7.71	0	8.59
6	7.16	7.38	0	8.97
Mean	7.57	7.66	0	8
STD	0.26	0.14	0	0.79

10

Figure 1 shows a graph of the effect of the inclusion of coconut endosperm fibre in the canine large intestine model on the survival of *Campylobacter jejuni*. Letters denote statistically significant difference ( $p < 0.05$ ).

15

Recorded pH after 24 hours incubation with coconut endosperm fibre included and omitted from the system for each dog.

Dog No.	+0.7% Coconut endosperm fibre	No Coconut endosperm fibre
1	6.75	7.5
2	6.25	7.5
3	6.75	7.5
4	6.25	7.5
5	6.25	7.5
6	6.25	7.5

At the end of the incubation period the pH of the solutions in each flask was measured and was found to be 7.5 when coconut endosperm fibre was omitted from the system (SD of 0). When coconut endosperm fibre was included in the model, the pH was found to be 6.42 (SD of 0.26).

### CONCLUSION

Inclusion of coconut endosperm fibre in a model of the canine large intestine resulted in the elimination of viable *Campylobacter jejuni* cells. With no coconut endosperm fibre added to the system, *Campylobacter jejuni* cells showed no loss in viability for the duration of the experiment. As a pH range of 6.5 to 7.5 is optimum for *Campylobacter*, it is unlikely that the difference in pH observed between the two conditions was responsible for the difference observed in survival. Instead, it is likely that the non-pathogenic, saccharolytic bacteria present in the faeces metabolise the coconut endosperm fibre. *Campylobacter jejuni* is incapable of fermenting carbohydrates, thus the coconut endosperm fibre being present gives the non-pathogenic, saccharolytic bacteria an advantage.

#### EXAMPLE 4

The effect of galactooligosaccharide (GOS) on the survival of *Salmonella enterica* serotype Typhimurium in a model of the canine large intestine.

- 5 • *Salmonella* is one of the most predominant gastrointestinal pathogens causing both clinical and non-clinical infections in dogs.
- Shedding of *Salmonella* in faeces can continue for 3 to 6 weeks increasing zoonotic risk in humans especially young children.
- 10 • An *in vitro* model of the canine large intestine has been developed to test the effect of non-digestible carbohydrates on the survival of canine bacterial pathogens.
- 15 • Inclusion of GOS in this model resulted in the elimination of viable *S. enterica* serotype Typhimurium cells from the system. This translates to a reduction in shedding times of *Salmonella* in a host animal.

20 Studies were undertaken to determine whether the inclusion of the prebiotic galactooligosaccharide (GOS) in an *in vitro* model of the canine large intestine would have any effect on the survival of a key pathogen of interest in dogs, *Salmonella*. GOS was obtained from Borculo Domo Ingredients and the product name is lactifit.

#### METHOD

25

1) *S. enterica* serotype Typhimurium (strain 7128) cells were grown from stock cultures and cultured at 37 °C. Liquid cultures were grown in 20 ml volumes in

50ml conical flasks shaken on an orbital shaker. Overnight cultures grown in Mueller Hinton (MH) broth (Oxoid) were adjusted to  $A_{600}$  1.0 before inclusion in the assay.

- 5 2) Flasks were set up with 200 ml MH broth, 1 ml of the adjusted *Salmonella* culture, and 2 g fresh faeces. To test flasks, 1.4 g GOS (CG86) was added and swirled to mix. Equivalent of 0.7 % w/v. Control flasks had no further additions.
- 10 3) Flasks were sampled at the start (0 hours) and end (24 hours) of the experiment to determine viable counts of *Salmonella* cells by serially diluting samples from the flasks and plating dilution's onto *Salmonella* selective agar (LabM). Plates were incubated for 48 hours after which, viable numbers were determined.
- 15 4) At the end of the experiment the pH of the mixture in each flask was determined using pH Boy (Camlab Limited, Nuffield Road, Cambridge, CB4 1TH).
- 20 5) The experiment was conducted six times using a faecal sample from a different dog each time. All dogs were fed a commercially available premium (complete and balanced) dry food.

## RESULTS

25 After a 24 hour microaerobic incubation, no viable *Salmonella* cells could be recovered from the flasks supplemented with GOS. In contrast, *Salmonella* cells were recovered from the flasks that contained no GOS at around  $10^7$  cells per ml. The results from the 6 individual experiments are shown in the table below and in Figure 2.

**Table 4** Number of viable *S. enterica* serotype Typhimurium cells ( $\log_{10}$ ) recovered from the model of the canine large intestine with and without the addition of GOS.

5 <— $\log_{10}$  colony forming units of *S. enterica* serotype Typhimurium—>

Faecal Sample	0 hours + 0.7% GOS	0 hours NO GOS	24 hours + 0.7% GOS	24 hours NO GOS
GR1	6.53	6.4	0	7.78
L1550	6.16	6.06	0	7.25
L136	6.47	6.48	0	7.86
L1502	6.25	6.09	0	7.32
L1515	6.41	6.3	0	7.11
GR3	6.51	6.25	0	7.77
Mean	6.39	6.26	0	7.52
STD	0.15	0.17	0	0.32

**Figure 2.** Graph to show the effect of the inclusion of GOS in the canine large intestine model on the survival of *S. enterica* serotype Typhimurium. Different letters denote statistically significant difference ( $p < 0.05$ ).

10

At the end of the incubation period the pH of the solutions in each flask was measured and was found to be 7.2 when GOS was omitted from the system (SD of 0.26). When GOS was included in the model, the pH was found to be 5.1 (SD of 0.17).

15

### CONCLUSION

Inclusion of GOS in a model of the canine large intestine resulted in the elimination of viable *Salmonella* cells. With no GOS added to the system, *Salmonella* cells were

able to survive for the duration of the experiment. It is likely that the non-pathogenic, saccharolytic bacteria present in the faeces metabolise the GOS and produce SCFA that decrease the pH in the model of the large intestine used in this study and this decrease in pH cannot be tolerated by *Salmonella*. Thus it is likely that the difference in pH observed between the two conditions was responsible for the difference observed in survival. *Salmonella* is capable of fermenting carbohydrates but it is unlikely that it is capable of fermenting GOS, thus the presence of GOS gives the non-pathogenic, saccharolytic bacteria an advantage. Saccharolytic bacteria increase in numbers and produce end products of fermentation that inhibit the growth of *Salmonella*.

#### Appendix 1

The Englyst method, from Englyst and Cummings (*Supra*).

#### Experimental

##### Apparatus

The fractionation procedure was carried out in 50-60ml screw-topped glass centrifuge tubes as previously described. Gas-liquid chromatography was performed with a Pye Unicam Series 204 chromatograph, fitted with a flame-ionisation detector. A 2.1m x 2mm i.d. glass column packed with Supelcoport (100-200 mesh) coated with 3% SP 2330 was used. The column temperature was 215°C (isothermal) and the injector and detector temperatures were 250°C. The carrier gas (nitrogen) flow-rate was 20ml min<sup>-1</sup>.



### Reagents

High purity certified reagents were used for all analyses. Enzyme preparations were as follows: hog pancreatic  $\alpha$ -amylase, E.C.3.2.1.1. (Sigma, Cat. No. A4268); pullulanase, E.C.3.2.1.41. (Boehringer, Cat. No. 108944).

5

### Method

The sequence of steps in the procedure is summarised below.

#### *Pre-treatment of sample*

10

As far as possible, foods should be analysed without any pre-treatment. If there are problems in taking a representative sample, foods with a low water content can be ball milled for 2-3 minutes, and those with a higher water content homogenised, or freeze-dried and ball milled.

15

#### *Sample Mass*

Accurately weigh between 50 and 1,000mg of sample, containing not more than 150mg of starch and 50mg of NSP, into a 50-60ml screw-top centrifuge tube and add a stirrer.

20

#### *Fat Extraction and Drying*

25

Samples with dry matter between 90 and 100% and with less than 20% of fat can be analysed directly. Otherwise, add 40ml of acetone, mix for 30 minutes by using a magnetic stirrer, centrifuge and remove by aspiration as much of the supernatant as possible without disturbing the residue. Place the tubes in a water bath at 65°C on a magnetic stirrer hot plate and mix the residue for a few minutes until it appears to be dry. The beaker can be covered and the acetone vapour removed by water pump.

*Dispersion of the Starch*

Add 2ml of DMSO, cap the tube and heat it in a boiling water bath for 1 hour, timed from when re-boiling commences, stirring continuously. Then, without cooling, add 8ml of 0.1M sodium acetate buffer pH5.2, at 50°C and vortex mix immediately.

5

50-200mg Sample

Add 2ml of DMSO

Heat for 1 hour at 100°C

10

Add 8ml of buffer, pH 5.2 and 0.1ml of enzyme solution

Incubate for 16 hours at 45°C

Add 40ml of EtOH

Leave for 1 hour, centrifuge

15

Wash twice with 85% EtOH, dry residue

Add 1ml of 12M  $H_2SO_4$

Leave 1 hour at 35°C

20

Add 11ml of  $H_2O$

Leave 2 hours at 100°C

Add 2ml of internal standard

25

13ml to uronic acids

1ml

30

Add 0.2ml of 12 M  $NH_3$ , 5 $\mu$ l of octan-2-ol and 0.1ml of  $NaBH_4$  solution

Leave for 1 hour at 40°C

Add 0.1ml of acetic acid

To 0.2ml add 0.3ml of Nmetlm and 2ml of acetic anhydride

5 Leave for 10 min at 20°C

Add 5ml of H<sub>2</sub>O, 1ml of CH<sub>2</sub>CL<sub>2</sub> and vortex

10 Use 1-2μl of the lower phase for GLC

Procedure for the analysis of non-starch polysaccharides (NSP)

*Enzyme Hydrolysis of the Starch*

15 Cool the tube to 45°C and immediately add 0.1ml of an enzyme solution containing 5,000 units of α-amylase and 5 units of pullulanase per ml of acetate buffer at pH 5.2. Incubate the samples at 45°C for 16-18 hours, preferably mixing continuously as described previously.

20 Following the enzyme treatment, add 40ml of absolute ethanol, mix well and leave to stand for 1 hour at room temperature. Centrifuge for 10 minutes or until a clear supernatant liquid is obtained. Removed by aspiration as much of the supernatant liquid as possible, without disturbing the residue, and discard it. Wash the residue twice with 50ml of 85% ethanol by mixing to form a suspension, centrifuging until clear and removing the supernatant liquid as before. Add 40ml of acetone to the  
25 washed residue, stir for 5 minutes and then centrifuge. Remove the supernatant liquid by aspiration and dry the residue as described under *Fat extraction and drying*.

*Acid hydrolysis of the residue from enzymic digestion*

Disperse the dried residue in 1ml of 12M sulphuric acid, using a vortex mixer. Leave at 35°C for 1 hour to solubilise the cellulose, then rapidly add 11ml of water and mix.

5

Heat the solution in a boiling water bath for 2 hours from re-boiling, stirring continuously. Cool it to room temperature by placing the tube in water, add 2ml of internal standard (2 mg of allose per ml of saturated benzoic acid solution) and mix the contents of the tube. Use 1ml of the hydrolysate for the preparation of alditol acetates and keep the remainder for the determination of uronic acids.

10

*Uronic acids*

The method used is a modification of the method of Scott. Mix 0.3ml of hydrolysate (diluted, if necessary, so that it contains between 25 and 100µg of uronic acids per ml) with 0.3ml of a mixture of sodium chloride-boric acid solution (prepared by adding 2g of sodium chloride and 3g of boric acid to 100ml of water) Add 5ml of concentrated sulphuric acid and vortex mix, then place the tube in a heating block at 70°C. Leave the tube and contents for 40 minutes and then cool them to room temperature by placing in water. When cool, add 0.2ml of 3,5-dimethylphenol solution (0.1g of  $(\text{CH}_3)_2\text{-C}_6\text{H}_3\text{OH}$  in 100ml of glacial acetic acid) and mix immediately. Between 10 and 15 minutes later read the absorbance at 400 and 450nm in a spectrophotometer against a water reference. Subtract the reading at 400nm from that at 450nm for each sample and plot the difference obtained for glucuronic acid standards (over the range 25-125µg ml<sup>-1</sup>). Read the sample concentrations from the graph.

15

20

25

*Preparation of alditol acetates*

To 1ml of hydrolysate add 0.2ml of 12M ammonia solution and 5 $\mu$ l of octan-2-ol. Test that the solution is alkaline, and then add 0.1ml of a freshly prepared solution of 100mg of sodium tetrahydroborate (III) (sodium borohydride) per ml of 3M ammonia solution. Mix, leave the mixture for 1 hour at 40°C and add 0.1ml of glacial acetic acid. Next, to 0.2ml of the acidified solution add 0.3ml of *N*-methylimidazole and 2ml of acetic anhydride, and mix. Leave it for 10 minutes at 20°C (room temperature), add 5ml of water, mix, and when cooled add 1ml of dichloromethane, agitate the contents vigorously on a vortex mixer and centrifuge for a few minutes to separate the mixture into two phases. Remove the bulk of the upper phase by aspiration and discard it, then transfer the lower phase to a small vial, seal and store it at -20°C. Use 1-2 $\mu$ l for injection on to the chromatograph.

*Alternative preparative of alditol acetates*

When dichloromethane is used as a solvent for the alditol acetates it has been observed in a number of laboratories without automatic GLC injection facilities that the injection technique is critical to the obtaining of reproducible results. A more robust method can be obtained if dichloromethane is replaced with ethyl acetate as a solvent for alditol acetates. The procedure is as follows:

To 1ml of hydrolysate add 0.2ml of 12M ammonia solution and 5 $\mu$ l of octan-2-ol. Test that the solution is alkaline, then add 0.1ml of a freshly prepared solution of 100mg of sodium tetrahydroborate (III) per ml of 3M ammonia solution. Mix, leave the mixture for 1 hour at 40°C and add 0.1ml of glacial acetic acid. To 0.5ml of the acidified solution add 0.5ml of *N*-methylimidazole, 5ml of acetic anhydride and mix. Leave for 10 minutes at 20°C (room temperature), then add 0.6ml of ethanol and mix. After 5 minutes add 5ml of water, place in a water bath at room temperature, add 5ml of 7.5M KOH and a few minutes later a further 5ml of 7.5M KOH. Mix by

inverting and leave to separate into two phases. Transfer the top phase to a small vial and store at +5°C. Use 1-2 $\mu$ l for injection on the chromatograph.

#### Other Embodiments

5

It is to be understood that, while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Other aspects, advantages, and modifications of the invention are within the scope of the claims set forth below.

CLAIMS

1. Use of a non-digestible carbohydrate in the manufacture of a composition for the prevention or treatment of pathogenic bacteria in the large intestine of a pet animal.
2. Use, as claimed in claim 1, wherein the non-digestible carbohydrate is a dietary fibre component.
3. Use, as claimed in claim 2, wherein the dietary fibre source is coconut endosperm fibre.
4. Use, as claimed in any one of the preceding claims, wherein the non-digestible carbohydrate is an oligosaccharide.
5. Use, as claimed in any one of the preceding claims, wherein the oligosaccharide is one of more of fructooligosaccharide, galactooligosaccharide, inulin, lactosucrose, maltooligosaccharide, xylooligosaccharide or raffinose.
6. Use, as claimed in any one of the preceding claims, wherein the pathogenic bacteria is *Campylobacter*, pathogenic *Escherichia coli* or *Salmonella*.
7. Use, as claimed in claim 6, wherein the pathogenic bacteria is the species *Campylobacter jejuni*, or the species *Salmonella enterica*.
8. Use, as claimed in any one of the preceding claims, wherein the pet animal is a dog or a cat.

9. Use, as claimed in any one of the preceding claims, wherein the composition is a pet food product.
10. A method for the prevention or treatment of pathogenic bacteria in the large intestine of a pet animal, the method comprising administering to said pet animal a composition which comprises a non-digestible carbohydrate.
11. A method as claimed in claim 10, wherein the dietary fibre source is one of coconut endosperm fibre, beet pulp, chicory, citrus pulp, rice bran, carob bean or gum talha.
12. A method as claimed in claim 10 or claim 11, wherein the non-digestible carbohydrate is an oligosaccharide.
13. A method as claimed in any one of claims 10 to 12, wherein the oligosaccharide is one of more of fructooligosaccharide, galactooligosaccharide, inulin, lactosucrose, maltooligosaccharide, xylooligosaccharide or raffinose.
14. A method, as claimed in any one of claims 10 to 13, wherein the pathogenic bacteria is *Campylobacter*, *Escherichia coli* or *Salmonella*.
15. A method, as claimed in claim 14, wherein the pathogenic bacteria is the species *Campylobacter jejuni* or the species *Salmonella enterica*.
16. A method, as claimed in any one of claims 10 to 15, wherein the pet animal is a dog or a cat.



17. A method, as claimed in any one of claims 10 to 16, wherein the composition is a pet food product.

5 18. A process for the manufacture of a pet food product as claimed in claim 9 or claim 17, comprising mixing together ingredients with a non-digestible carbohydrate with optional heating/cooking and forming a pet food product

1 / 1

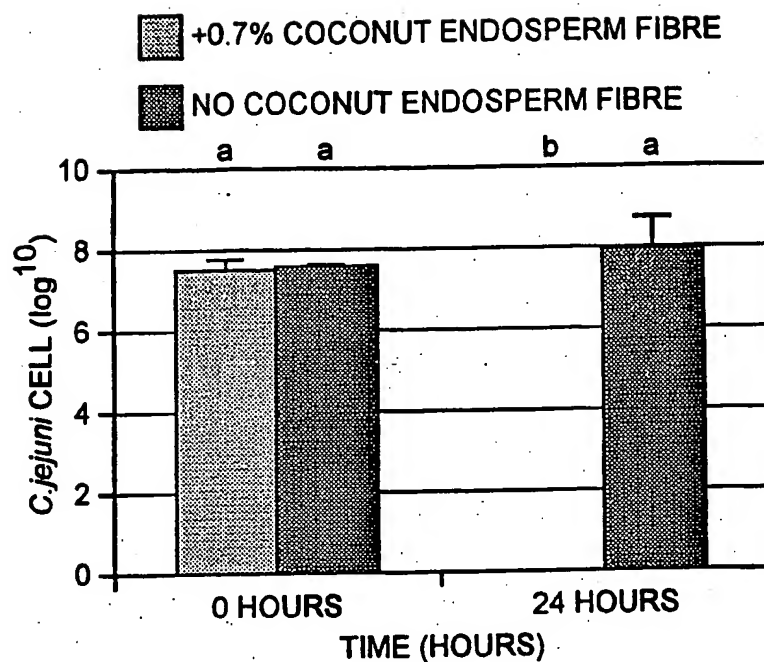


FIG. 1

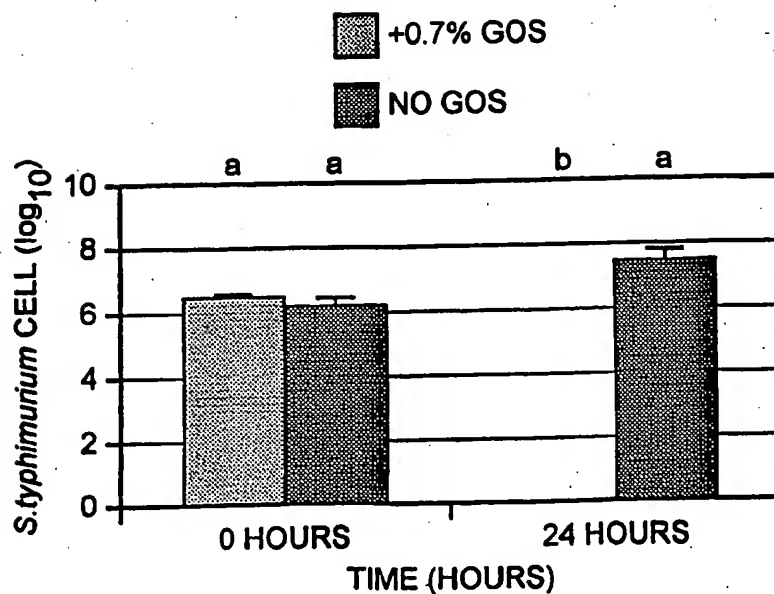


FIG. 2

Int. Application No  
PCT/GB 01/01036

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7      A23K1/14      A23K1/18      A23K1/16      A61P1/12      A61K35/78

### B. FIELDS SEARCHED

IPC 7 A23K A61K

EPO-Internal, PAJ, CHEM ABS Data, BIOSIS, FSTA, WPI Data

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

'&' document member of the same patent family

**23 August 2001**

30/08/2001

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European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel (+31-70) 340-2040, Tx. 31 651 epo nl,  
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# INTERNATIONAL SEARCH REPORT

Int. Application No.  
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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

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